

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

230-148P

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/509945

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/JP99/04167

August 3, 1999

August 4, 1998

**TITLE OF INVENTION**

MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

**APPLICANT(S) FOR DO/EO/US**

HAMADA, Kazuyuki; NAKAKIDO, Fumio

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19<sup>th</sup> month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

1. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.-1449 and International Search Report (PCT/ISA/210)
2. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
3. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
4. ☐ A substitute specification.
5. ☐ A change of power of attorney and/or address letter.
6. ☒ Other items or information:
  - 1.) PCT Request (PCT/RO/101)
  - 2.) Sequence Listing (12 pages)
  - 3.) Verification of Translation
  - 4.) Zero (0) sheets of Formal Drawings

745

PCT/JP99/04167

ATTORNEY'S DOCKET NUMBER 230-148P  
14 APR 2003

The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):**  
 either international preliminary examination fee (37 CFR 1.482)  
 nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO  
 and International Search Report not prepared by the EPO or JPO. . . . . \$970.00  
  
 International preliminary examination fee (37 CFR 1.482) not paid to  
 USPTO but International Search Report prepared by the EPO or JPO . . . . . \$840.00  
  
 International preliminary examination fee (37 CFR 1.482) not paid to USPTO  
 but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. . . . . \$690.00  
  
 International preliminary examination fee (37 CFR 1.482) paid to USPTO  
 but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . \$670.00  
  
 International preliminary examination fee (37 CFR 1.482) paid to USPTO  
 and all claims satisfied provisions of PCT Article 33(1)-(4). . . . . \$96.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**  
 Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30  
 months from the earliest claimed priority date (37 CFR 1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	30 - 20 =	10	X \$18.00
Independent Claims	5 - 3 =	2	X \$78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable) Yes			+ \$260.00

**TOTAL OF ABOVE CALCULATIONS =**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement  
 must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

**SUBTOTAL =**

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30  
 months from the earliest claimed priority date (37 CFR 1.492(f)).

**TOTAL NATIONAL FEE =**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
 accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

**TOTAL FEES ENCLOSED =**

Amount to be: refunded	\$
charged	\$

- a. ☒ A check in the amount of \$ 1476.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account. No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees.  
 A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any  
 overpayment to Deposit Account No. 02-2448.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:

**Birch, Stewart, Kolasch & Birch, LLP** or Customer No. 2292  
 P.O. Box 747  
 Falls Church, VA 22040-0747  
 (703)205-8000

SIGNATURE

*for*  
**MURPHY, GERALD M., JR.**  
 NAME

**#28.977 (GMM)**  
 REGISTRATION NUMBER

QC

09/509945  
527 Rec'd PCT/PTO 04 APR 2000

PATENT  
230-148P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: HAMADA, Kazuyuki et al.  
Int'l. Appl. No.: PCT/JP99/04167  
Appl. No.: New Group:  
Filed: April 4, 2000 Examiner:  
For: MUTANT BARNASE GENE AND TRANSGENIC  
PLANT TRANSFORMED BY SAID GENE

PRELIMINARY AMENDMENT

**BOX PATENT APPLICATION**

Assistant Commissioner for Patents  
Washington, DC 20231

April 4, 2000

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/JP99/04167 which has an International filing date of August 3, 1999, which designated the United States of America.--

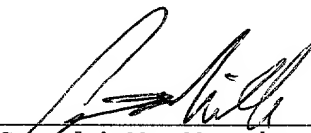
REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By  #32868  
Gerald M. Murphy, Jr., #28,977

GMM/cqc  
230-148P

P.O. Box 747  
Falls Church, VA 22040-0747  
(703) 205-8000

(Rev. 01/08/2000)

## SPECIFICATION

MUTANT BARNASE GENE AND TRANSGENIC  
PLANT TRANSFORMED BY SAID GENE

The present application claims priority from Japanese  
5 Patent Application No.: Hei 10-220060, the disclosure of  
which is incorporated herein by reference.

## FIELD OF THE INVENTION

This invention relates to a mutant barnase gene which  
makes it possible to efficiently yield a male sterile  
10 transgenic plant when expressed in a specific site of a  
plant, in particular, anther-specifically. The present  
invention further relates to a recombinant vector capable of  
expressing the mutant barnase gene of the invention in host  
cells, a plant transformed by this vector and a method for  
15 constructing a transgenic plant.

## PRIOR ART

Barnase is an RNase originating in *Bacillus*  
*amyloliquefaciens* (S. Nishimura and M. Nomura, Biochem.  
Biophys. Acta 30, 430-431:1958; R.W. Hartley, J. Mol. Biol.,  
20 202, 913-915:1988). This enzyme has 110 amino acid residues  
and hydrolyzes RNA. When expressed in cells, this enzyme  
inhibits the functions of the cells as a result of its  
potent RNase activity and thus causes cell death in many  
cases. By using this characteristic, it is therefore  
25 expected that the function of the specific site can be  
selectively controlled by expressing the barnase gene in a  
specific site of a plant.

PCT International Publication WO89/10396 discloses a

technique whereby a male sterile plant is obtained by constructing a male sterility gene by ligating the above-described barnase gene to the downstream of an anther tapetal cell-specific expression promoter and introducing  
5 the thus obtained gene into a plant. Male sterilization techniques are of great value in efficiently developing an F1 hybrid variety.

When the barnase gene is employed as a male sterility gene, however, it is frequently observed that resulting male  
10 sterile transgenic plants exhibit unfavorable characteristics. PCT International Publication WO96/26283 refers to this problem in rice. It is also reported that similar phenomena are observed not only in rice but in lettuce (Scientia Horticulturae 55, 125-139:1993; Arlette Reymaerts,  
15 Hilde Van de Wiele, Greta De Sutter, Jan Janssens: Engineered genes for fertility control and their application in hybrid seed production). According to this report, a plant with depressed activity was constructed by introducing a male sterility gene comprising a tobacco anther-specific  
20 promoter (TA29) and a barnase gene into lettuce.

Although reasons for these phenomena have not been accurately clarified so far, it is assumed that the so-called "position effect" of the site in which the gene is transferred may account for the mechanism (PCT International  
25 Publication WO96/26283). More specifically, a desired male sterile plant should be constructed if the male sterility gene is expressed exclusively in the target site (i.e., anthers). However, there is a possibility that the barnase

might be expressed also in tissues other than anthers  
although in a very small dose under the action of expression  
regulators (for example, an endogenous enhancer) existing in  
the vicinity of the gene transfer site. In such a case, the  
5 unfavorable characteristics as described above can appear  
because barnase has a strong enzymatic activity.

To overcome this problem, the method disclosed in PCT  
International Publication WO96/26283 exploits the character  
of cauliflower mosaic virus 35S promoter (hereinafter  
10 referred to as CaMV35S promoter) of being expressed potently  
in tissues other than anthers. Namely, barstar, i.e., an  
inhibitory protein against barnase is employed therein. The  
barstar gene ligated to a CaMV35S promoter is transferred  
into a plant simultaneously with a barnase gene and then the  
15 barstar gene is constitutively expressed in tissues other  
than the anthers, thereby eliminating the effects of the  
barnase in tissues other than the anthers. However, it is  
necessary in this method to transfer not only a barnase gene  
but also a barstar gene, and hence, the problem is that the  
20 application of this technique to the breeding of F1  
varieties of rice or corn seeds may give rise to a gene  
silencing. "Gene silencing" is a phenomenon wherein the  
expression of a gene is inhibited when plural copies of the  
foreign gene are introduced. It is known that this problem  
25 frequently occurs when the expression of a foreign gene is  
effected by a 35S promoter, though the detailed mechanism  
thereof has not been clarified yet (R.B. Flavell, Proc. Natl.  
Acad. Sci. USA 91, 3490-3496:1994; J. Finnegan, Bio.

Technology 12, 883-888:1994; M.A. Matzke and A.J.M. Matzke, Plant Physiol., 107, 679-685:1995). Since, in rice and corn, a barstar gene is used as a "fertility-restoring gene" in the pollen parent (father) so as to allow the pollen in the F1 generation to restore the fertility (C. Mariani, et al., Nature 357, 384-387:1992), if an MS plant (mother) is constructed by the method with the use of a barstar gene as reported in WO96/26283, the F1 plant has plural copies of the barstar gene. In such a case, there is a possibility that the expression of the gene may be inhibited due to the gene silencing.

#### SUMMARY OF THE INVENTION

The present invention provides a method for constructing a male sterile plant by using a barnase gene without resort to a barstar gene.

The present invention further provides a mutant barnase gene to be used in the above method and a process for producing the same.

#### DETAILED DESCRIPTION OF THE INVENTION

In the present invention, the DNA sequence of barnase gene (R.W. Hartley, J. Mol. Biol. 202, 913-915:1988) is mutated at least in part and then the thus obtained mutant barnase gene is anther-specifically expressed in a plant so as to make the plant substantially male sterile without any substantially disadvantageous effect on the tissues other than anthers.

The mutation can be performed by a known method such as site-specific mutagenesis, deletion of a fragment by

using restriction enzymes or the low fidelity PCR method. Among all, it is preferred to use the low fidelity PCR method. This method is described in detail in D. Leung, E. Chen and D. Goedda, Technique 1, 11-15:1989; Y.Z.

5 Xiaoping and R.H. Ebright, Nucleic Acid Res. 19, 6052:1991; G.C. Rice et al., Proc. Natl. Acad. Sci. USA 89, 5467-5471: 1992 and the contents of these documents are incorporated herein by reference. By using this technique, PCR is  
10 carried out under such conditions as to induce some errors during the amplification reaction. Thus, random mutations can be introduced into the target DNA fragment.

Primers to be used in the low fidelity PCR method in the present invention are selected as in the conventional PCR method. It is preferred that each of these primers has  
15 a similar number of nucleotides as in the conventional PCR method.

The present inventors performed PCR by using a DNA containing the sequence represented by SEQ ID NO:1 as a template with the use of a combination of primer 1 (5'-  
20 CGTTCGGCTC GATGGTACCG GTTATCAACA CGTTTGA-3': SEQ ID NO:6) and primer 2 (5'-CCTCTAGATT ATCTGATTTT TGTAAGGTC TGATAATG-3': SEQ ID NO:7) under such conditions as to induce errors. Thus, a mutant barnase gene having the DNA sequence represented by SEQ ID NO:3 was isolated. The sequence  
25 represented by SEQ ID NO:1 employed herein is the sequence of the coding region of the barnase gene contained in the known plasmid pVE108 (W092/13956). This sequence corresponds to the wild type barnase gene from which the

unnecessary moiety corresponding to the secretion signal in the N-terminal side has been deleted.

It is also possible to acquire different mutant barnase genes by a similar method.

5       The PCR amplification product is cloned in a host (for example, *Escherichia coli*) and clones containing a mutant barnase gene is isolated. In this cloning, clones having a mutant barnase gene may be screened by assaying the RNase activity expressed by the gene. However, it is advantageous  
10      to screen the clone by the method consisting of the following two steps by taking advantage of the fact that the RNase activity of barnase affects the growth of *E. coli*.

          In the first step, a plasmid is prepared by using the mutant barnase gene obtained above and then *E. coli* is  
15      transformed thereby. The growth of the *E. coli* transformant thus obtained will be inhibited by the activity of the mutant barnase. Based on this fact, a colony growing slowly (i.e., a small colony), compared with *E. coli* having a control vector free from any barnase gene, is selected.  
20      It is expected that the thus selected *E. coli* strain will contain the mutant barnase gene. To confirm that the inhibition of the growth of *E. coli* is due to the expression of the mutant barnase gene, the second step is then carried out.

25       In the second step, a barstar gene is employed. As described above, barstar is an inhibitory protein against barnase. In *E. coli* which expresses barstar, the enzymatic activity of barnase is inhibited and thus the degradation of

mRNA, which otherwise takes place in the presence of barnase, can be inhibited. Therefore if, *E. coli* which expresses a barstar gene is transformed with a barnase gene, the growth will not be inhibited. Accordingly, it is expected that the  
5 thus obtained *E. coli* transformant will produce little difference in growth rate from the transformant constructed by the control plasmid which is free from any barnase gene and, therefore, these transformants will produce little difference in colony size too. Based on this principle, a  
10 plasmid is prepared from the *E. coli* strain selected in the first step. The plasmid is then used to transform another *E. coli* strain, in which the barstar gene is constitutively expressed. Thus, mutant barnase gene-containing colonies having almost the same size as the one of the *E. coli*  
15 transformed by the control plasmid are selected. The *E. coli* with the constitutive expression of the barstar gene to be used herein can be prepared by, for example, the method as will be described in Example 1 hereinafter.

The DNA sequence of the thus obtained mutant barnase  
20 gene may be analyzed by a conventional method, if necessary, to thereby examine the mutation in detail.

A preferred example of the mutant barnase gene of the present invention is one which has the DNA sequence represented by SEQ ID NO:3. Compared with the nucleotide  
25 sequence of SEQ ID NO:1 coding for the wild type activity, the nucleotide sequence encoding this gene has an insertion of "T" at the 15-position from A in the initiation codon ATG and a deletion of "A" at the 333-position. According to the

normal translation manner, translation will be initiated from ATG at the 1-position of this DNA sequence represented by SEQ ID NO:3, and terminated at the ninth codon which has been converted to a termination codon as a result of the  
5 insertion of the "T" at the 15-position. It is unlikely that the thus formed oligopeptide consisting of 8 amino acids has the barnase activity. Further, there is no other ATG or GTG codon from which the translation can be initiated in the correct frame in the vicinity thereof. However, the  
10 fact that the growth rate of *E. coli* was restored by the barstar protein strongly suggests that the translation of the barnase protein from the gene of SEQ ID NO:3 proceeded in the correct frame. This is seemingly due to a phenomenon called "frame shift re-coding" whereby the ribosome controls  
15 the translation while automatically shifting the reading frame during the course of translation into the protein. This phenomenon, which has been reported in several genes of viruses, *E. coli* and animals, generally depends exclusively on an individual nucleotide sequence and neither any special  
20 protein nor translation device is required. The frame shifting efficiency during the translation varies from gene to gene, i.e., ranging from several % to 50%. In the case of the mutant barnase gene, it is considered that the efficiency of the shifting of the reading frame during the  
25 translation is not high and thus the translation product in the correct frame is formed in a smaller amount, thereby lowering the activity of the gene.

Moreover, there is a high possibility that other

mutant barnase genes of which the translation efficiency is lowered due to the shifting of the reading frame, as in the case of the DNA sequence represented by SEQ ID NO:3, or some other reasons can be obtained by mutating the barnase gene by, for example, the low fidelity PCR method. Furthermore, there is a high possibility that a gene having a DNA sequence derived from the DNA sequence represented by SEQ ID NO:3 by substitution, deletion, insertion or addition of one or more nucleotides will be a mutant barnase gene which provides a lowered translation efficiency due to the shifting of the reading frame as a result of the insertion of T at the 15-position from A in the initiation codon ATG and/or the deletion of A at the 333-position, as in the DNA sequence represented by SEQ ID NO:3. It is considered that each of these genes, likewise the gene of SEQ ID NO:3, is capable of making a plant substantially male sterile when it is anther-specifically expressed in the plant. Accordingly, these genes are also included in the scope of the present invention similar to the gene of SEQ ID NO:3.

The plant which is to be male sterilized by the mutant barnase gene is not particularly limited, so long as the gene can be transferred into the plant to provide a male sterilized plant. Examples of the plants include rice, corn, tobacco, lettuce and Brassica. Among all, rice and corn are preferred.

In order to male sterilize a plant, the mutant barnase gene is expressed specifically in the anthers of the plant to inhibit the anther functions by the RNase activity of

barnase. Such an anther-specific expression can be achieved by using the method described in WO92/13957. In brief, the mutant barnase gene is ligated to the downstream of an anther-specific promoter and then incorporated into plant  
5 cells with the use of an expression vector.

The incorporation may be performed by using the Agrobacterium method, the electroporation method, the particle gun method, etc.

From the transformed plant cells, a complete plant can  
10 be formed by the regeneration from a callus of the transformed plant cells in accordance with, for example, the method reported in Y. Hiei et al., Plant J. 6, 271-282:1994.

The male sterility of the thus constructed plant can be confirmed by the inability of the plant to fertilize  
15 unless it is pollinated from another fertilizable plant.

#### Example 1: Preparation of mutant barnase gene

##### Low fidelity PCR

Primer 1 (CGTTCGGCTC GATGGTACCG GTTATCAACA CGTTTGA: SEQ ID NO:6) and Primer 2 (CCTCTAGATT ATCTGATTTT TGTAAGGTC  
20 TGATAATG: SEQ ID NO:7) were synthesized by a DNA synthesizer (manufactured by Applied Biosystems) in accordance with the method described in S.L. Beaucage et al., Tetrahedron Lett., 22. 1859-1862:1982. Known plasmid pVE108 (WO92/13956) was used as a template and PCR was carried out with the use of  
25 the combination of Primers 1 and 2. SEQ ID NO:1 shows the sequence of the coding region of the barnase gene which is contained in pVE108. The reaction was performed for 50 cycles with each cycle consisting of 1 minute at 94°C,

1 minute at 57°C and 1 minute at 72°C in 10 mM Tris-HCl (pH 9.5), 50 mM KCl, 2 mM MgCl<sub>2</sub>, and 1 mM each of dNTP, 10 ng of the template DNA and 0.5 U of Taq DNA polymerase.

After the completion of the reaction, the amplification product was separated by electrophoreses on agarose gel (2% SeaKem GTG agarose, 1xTAE) and purified by the DEAE-cellulose method (M. Muramatsu, Labo-manual Idenshikogaku (Gene Engineering Labo-Manual), Maruzen, pp.111:1988). By using the purified product thus obtained as a template, PCR was performed again under the conditions as defined above.

Ligation of mutated barnase gene fragment into plasmid vector

The reaction product from the above step was digested with SacI and XbaI in a conventional manner and then purified by agarose gel electrophoresis to give an "insertion fragment". A plasmid to be used for transferring this insertion fragment into *E. coli* can be appropriately selected. For example, use may be made of plasmid pHM1 therefor. The plasmid pHM1 was constructed as follows.

Plasmid pBR322 was cleaved at the EcoRI site and blunt-ended by using T4 DNA polymerase (manufactured by Takara Shuzo). Separately, lacZ expression cassette (322 bp) was excised from pUC18 with PvuII and the ends thereof were blunted. The cassette was integrated into the restriction site of the blunt-ended plasmid pBR 322 to give plasmid pHM1.

Plasmid pHM1 was digested with SacI and XbaI followed by dephosphorylation with calf intestine alkaline phosphatase (manufactured by Takara Shuzo) to give a

restriction enzyme-treated plasmid fragment. Into this restriction enzyme-treated plasmid pHM1, the above "insertion fragment" containing a barnase gene was ligated by using Takara Ligation Kit ver.1 (manufactured by Takara Shuzo).

Introduction into *E. coli* and selection of barnase-active clone

The *E. coli* having the barnase gene introduced thereinto may be selected by, for example, the following method. Since mRNA is degraded in cells by the barnase activity, the synthesis of protein is suppressed and, in its turn, the growth of *E. coli* is inhibited. Thus, the *E. coli* transformed by the barnase gene can be selected by taking advantage of the fact that the desired transformant colony has a smaller size than the *E. coli* colony transformed by the control plasmid free from the mutant barnase gene. When the wild type barnase or a mutant barnase still maintaining the activity comparable thereto is integrated into pHM1, *E. coli* cannot form any colony. Thus, clones having a sufficiently weakened barnase activity can be exclusively selected.

Thus, the plasmid having the mutant barnase gene ligated thereto was precipitated from ethanol and then introduced into *E. coli* LE392 strain by the electroporation method with the use of GenePulser (BioRad) in accordance with the manufacture's instruction. Also, the control plasmid free from mutant barnase was introduced into the *E. coli* LE392 strain in the same manner.

Next, these *E. coli* transformants were plated onto an LB agar medium containing tetracycline (25 µg/ml) and incubated at room temperature (25°C) for 72 hours. Then colonies of a smaller size than the colony of *E. coli* having the control plasmid pHM1 introduced therein were screened as colonies of the *E. coli* transformants having the mutant barnase transferred therein (Table 1, A).

#### Selection of mutant barnase clone by barstar gene

As described above, barstar is an enzyme acting as an antagonist to barnase. In *E. coli* wherein barstar is expressed, the enzymatic activity of barnase is inhibited and thus the degradation of mRNA, which otherwise takes place in the presence of barnase, can be inhibited. Therefore, if *E. coli* expressing barstar is transformed by a plasmid containing a barnase gene, the growth thereof will not be inhibited. Accordingly, it is expected that the thus obtained *E. coli* transformant will show little difference in growth rate from the transformant constructed by using the control plasmid free from a barnase gene and, therefore, these transformants will show little difference in colony size too.

An *E. coli* transformant which expressed barstar was constructed in the following manner. A barstar gene described in R.W. Hartley, J. Mol. Biol. 202, 913-915:1998 was excised with HindIII and XbaI and ligated in frame to a tac promoter (de Boer et al., Proc. Natl. Acad. Sci. USA 80, 21-25:1983). The gene, together with a chloramphenicol tolerance gene (N.K. Alton and D. Vapnek, Nature 282, 864-

869:1979), was ligated into the defective transposon which lacked the transferase in a vector described in Herrero et al., J. Bacteriol. 172, 6557-6567:1990. Subsequently, the thus obtained plasmid was introduced into *E. coli* MC1061 strain thereby to transfer the transposon, which contained the barstar gene cassette, into the chromosome of the *E. coli*. It was confirmed based on the chloramphenicol tolerance that this *E. coli* was able to maintain the barstar gene cassette in a stable state. Since the *E. coli* MC1061 strain lacked the *lacI* gene, the *tac* promoter was continuously active and thus the barstar gene was constitutively expressed.

The plasmid containing the mutant barnase gene as the insertion fragment and the control plasmid free from the same were each introduced into the above *E. coli* transformant by the electroporation method. Then the *E. coli* transformants were plated onto an LB agar medium containing IPTG (1mM) and tetracycline (25 µg/ml) and incubated at 25°C for 72 hours. Among the colonies of the *E. coli* transformed by the mutant barnase gene, a colony being comparable in size to the colony of the *E. coli* having the control plasmid pHM1, which had been simultaneously plate-cultured, was screened and named "#4-31" (Table 1, B).

Table 1: Screened clone and colony size (mm)

	Screened clone	pHM1 (control)
A: <i>E. coli</i> LE392* <sup>1</sup>	1.77 ± 0.33	2.65 ± 0.47
B: <i>E. coli</i> with barstar gene expression* <sup>2</sup>	1.39 ± 0.10	1.55 ± 0.08

\*1: Colony size after incubation on the LB agar plate containing 25 µg/mL of tetracycline at 28°C for 48 hours.

5 \*2: Colony size after incubation on the LB agar plate containing 25 µg/mL of tetracycline at 28°C for 24 hours.

Example 2: Determination of nucleotide sequence of mutant barnase gene

10 From the *E. coli* screened in Example 1, a cloned mutant barnase gene appropriate for the object of the present invention was prepared. First, the clone #4-31 screened in Example 1 was propagated. Next, the mutant barnase gene fragment in the clone #4-31 was excised with

15 KpnI and XbaI and ligated into the KpnI, XbaI-site of pUC119 having been cleaved similarly with KpnI and XbaI. After amplifying the obtained plasmid by using *E. coli*, a reaction was performed by the cycle sequence method with the use of

20 Taq polymerase (Taq Dye Terminator Cycle Sequencing Kit, manufactured by Applied Biosystems Inc.) in accordance with the manufacturer's protocol. Subsequently, the sequence was analyzed with a DNA Sequencer (Model 373A, manufactured by Applied Biosystems Inc.), thereby yielding the sequence represented by SEQ ID NO:3. Compared with the DNA sequence

of the wild type barnase gene, this sequence had an insertion of T at the 15-position from A of the initiation codon ATG and a deletion of A at the 333-position.

Example 3: Construction of male sterile rice by using  
5 attenuated mutant barnase gene

The attenuated mutant barnase gene integrated into pUC119 as described above was cleaved with restriction enzymes XbaI and KpnI and thus the plasmid vector pTS431 represented by SEQ ID NO:5 was constructed. Although this  
10 plasmid pTS431 differs from the known plasmid pVE108 (PCT International Publication WO92/13956) in the following points, it is substantially equivalent thereto except for the moieties of the anther-specific promoter and the mutant barnase.

15 (1) In the plasmid vector pTS431 according to the present invention, the barnase gene (SEQ ID NO:1) in the known plasmid pVE108 has been converted into the mutant barnase gene (SEQ ID NO:3).

(2) A tobacco-origin anther specific promoter is used  
20 in the known plasmid pVE108, while the anther specific promoter of rice E1 gene (PCT International Publication WO92/13956) is used in the plasmid vector pTS431 according to the present invention.

(3) In the present invention, a 35S3 promoter (EP  
25 0344029) of 1376 bp, which is not used in the plasmid pVE108, is employed upstream of the barnase gene.

(4) In the present invention, a sequence originating in the downstream of Agrobacterium T-DNA gene 7 excised from

pJD884 (PCT International Publication WO93/09218) is employed whereas such a sequence is not used in the plasmid pVE108.

(5) In the present invention, the region corresponding to lacZ has been deleted from the pUC19-origin moiety. In addition a plasmid (pTS172) having the wild type barnase gene integrated therein is represented by SEQ ID NO:4.

The fragment of about 4.5 kbp was excised with a restriction enzyme EcoRI respectively from pTS431 (a plasmid having the mutant barnase gene transferred thereinto; SEQ ID NO:5) and pTS172 (a plasmid having the barnase gene transferred thereinto; SEQ ID NO:4) and inserted into the EcoRI-site of the intermediate vector pSB11 (T. Komari et al., Plant J. 10(1), 165-174:1996), and further, the T-DNA region thereof was integrated into the acceptor vector pSB1 (T. Komari et al., Plant J. 10(1), 165-174:1996) by way of homologous recombination. By using *Agrobacterium tumefaciens* LBA4404 having the obtained recombinant plasmids (pSB1431 and pSB1172 respectively), rice (variety: Asanohikari) was transformed. While the transformation was performed basically in accordance with the method of Hiei et al. (Plant J. 6(2), 271-282:1994), phosphinothricine (concentration: 10 mg/L) was employed for screening transformants since the thus constructed male sterility genes contained bar gene encoding phosphinothricine acetyl transferase as a selective marker. Phosphinothricine facilitates selection of calluses having the gene transferred thereinto.

A comparison of the rice transformant obtained with the use of the wild type barnase gene with the one obtained with the use of the mutant barnase gene indicates that the transfer of the mutant barnase gene gave rise to a

5 remarkable improvement in the transformation efficiency and an increase in the rate of morphologically normal male sterility transformants, as shown in Table 2.

Table 2: Transformation efficiency

	No. of infected calluses	No. of regenerated calluses	No. of PCR-positive lines <sup>*1</sup>	No. of morphologically normal male sterile lines
pSB1172 (control)	2838	83	52/83	9/52 (17.3%)
pSB1431 (present invention)	787	69	43/45 <sup>*2</sup>	27/28 <sup>*3</sup> (96.4%)

\*1: detection of barnase gene fraction by PCR.

10 \*2: examined only 45 lines among 69.

\*3: examined only 28 lines among 43.

#### Effects of the Invention

In the present invention, a barnase gene having a weakened effect is constructed via mutation. A male

15 sterility gene comprising the mutant barnase gene can be introduced into a plant to successfully produce a male sterile plant free from any unfavorable characteristic at a high efficiency with the use of a single gene without resort to the use of a barstar gene.

## CLAIMS

1. A mutant barnase gene having one or some mutations at least in part of the DNA sequence of barnase gene, wherein said mutant barnase gene is capable, when anther-specifically expressed in a plant, of making said plant substantially male sterile without exerting any substantially disadvantageous effect on the tissues except for the anthers.
2. The gene as claimed in Claim 1, wherein said mutation is a mutation causing frame shift re-coding.
3. A gene which is derived from a DNA sequence encoding the same amino acid sequence as shown in SEQ ID NO:1 by substitution, deletion, insertion or addition of one to several nucleotides in said DNA sequence, wherein said gene is capable of encoding a protein which makes a plant substantially male sterile when expressed anther-specifically in said plant.
4. A gene comprising a DNA sequence which is derived from a DNA sequence encoding the same amino acid sequence as shown in SEQ ID NO:1 by substitution, deletion, insertion or addition of one to several nucleotides in the latter DNA sequence, and a promoter located upstream of said DNA sequence for allowing an anther-specific expression, wherein said gene is capable of making a plant substantially male sterile when introduced into the genome of said plant.
5. A gene represented by SEQ ID NO:3 which encodes a protein capable of making a plant substantially male sterile when expressed anther-specifically in said plant.

6. A gene comprising the sequence represented by SEQ ID NO:3 and a promoter located upstream of said sequence for allowing an anther-specific expression, wherein said gene is capable of making a plant substantially male sterile when introduced into the genome of said plant.

7. A recombinant vector which contains a gene as claimed in any of Claims 1 to 6 and expresses said gene in a host plant.

8. A method of making a plant male sterile which comprises transforming said plant by a mutant barnase gene as claimed in any of Claims 1 to 6 and allowing said mutant barnase gene to be expressed anther-specifically.

9. The method as claimed in Claim 8, wherein said plant is transformed by said mutant barnase gene by integrating said gene into the genome of said plant.

10. A transgenic plant wherein a gene as claimed in any of Claims 1 to 6 has been introduced.

# ABSTRACT

A male sterile plant free from any undesirable characteristic is constructed by anther-specifically expressing barnase gene alone. A barnase gene, which has a mutation and thus sustains its activity at a lowered level, is transferred into a plant and anther-specifically expressed, thereby efficiently providing a male sterile transgenic plant.

# BIRCH, STEWART, KOLASCH & BIRCH, LLP

## COMBINED DECLARATION AND POWER OF ATTORNEY

ATTORNEY DOCKET NO.

230-148P

### FOR PATENT AND DESIGN APPLICATIONS

PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:



Insert Title:

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor ( if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:  
**MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE**

Fill in Appropriate  
Information -  
For Use Without  
Specification  
Attached:

the specification of which is attached hereto. If not attached hereto,  
the specification was filed on \_\_\_\_\_ as  
United States Application Number \_\_\_\_\_; and /or  
the specification was filed on August 3, 1999 as PCT  
International Application Number PCT/JP99/04167; and was  
amended under PCT Article 19 on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Insert Priority  
Information:  
(if appropriate)

➔ Prior Foreign Application(s)

(Number)	(Country)	(Month/Day/Year Filed)	Priority Claimed
<u>220060/1998</u>	<u>Japan</u>	<u>8/4/1998</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

Insert Provisional  
Application(s):  
(if any)

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
_____	_____
_____	_____

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months (6 Months for Designs) Prior To The Filing Date of This Application:

Insert Requested  
Information:  
(if appropriate)

Country	Application No.	Date of Filing (Month/Day/Year)
_____	_____	_____
_____	_____	_____

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Insert Prior U.S.  
Application(s):  
(if any)

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
_____	_____	_____
_____	_____	_____

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

Terrell C. Birch	(Reg. No. 19,382)	Raymond C. Stewart	(Reg. No. 21,066)
Joseph A. Kolasch	(Reg. No. 22,463)	James M. Slattery	(Reg. No. 28,380)
Bernard L. Sweeney	(Reg. No. 24,448)	Michael K. Mutter	(Reg. No. 29,680)
Charles Gorenstein	(Reg. No. 29,271)	Gerald M. Murphy, Jr.	(Reg. No. 28,977)
Leonard R. Svensson	(Reg. No. 30,330)	Terry L. Clark	(Reg. No. 32,644)
Andrew D. Meikle	(Reg. No. 32,868)	Marc S. Weiner	(Reg. No. 32,181)
Joe McKinney Muncy	(Reg. No. 32,334)	Andrew F. Reish	(Reg. No. 33,443)
C. Joseph Faraci	(Reg. No. 32,350)	Donald J. Daley	(Reg. No. 34,313)

Send Correspondence to:

**BIRCH, STEWART, KOLASCH & BIRCH, LLP**

**P.O. Box 747 • Falls Church, Virginia 22040-0747**

**Telephone: (703) 205-8000 • Facsimile: (703) 205-8050**

PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor: Insert Name of Inventor Insert Date This Document is Signed Insert Residence Insert Citizenship Insert Post Office Address	GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
	Kazuyuki HAMADA		Kazuyuki Hamada	March 13, 2000
	Residence (City, State & Country) Shizuoka, Japan		CITIZENSHIP Japanese	
Full Name of Second Inventor, if any see above	GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
	Fumio NAKAKIDO		Fumio Nakakido	March 13, 2000
	Residence (City, State & Country) Shizuoka, Japan		CITIZENSHIP Japanese	
Full Name of Third Inventor, if any see above	POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438-0802 Japan			
	GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Full Name of Fourth Inventor, if any see above	POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438-0802 Japan			
	GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Full Name of Fifth Inventor, if any see above	POST OFFICE ADDRESS (Complete Street Address including City, State & Country) c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438-0802 Japan			
	GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*

## SEQUENCE LISTING

&lt;110&gt; Japan Tobacco Inc.

&lt;120&gt; MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

&lt;130&gt; 980687

&lt;160&gt; 7

&lt;210&gt; 1

&lt;211&gt; 343

&lt;212&gt; DNA

<213> Bacillus amyloliquefaciens

&lt;220&gt;

&lt;221&gt; NAME/KEY: CDS

&lt;222&gt; 1..336

&lt;400&gt; 1

atg gta ccg gtt atc aac acg ttt gac ggg gtt gcg gat tat ctt cag 48

Met Val Pro Val Ile Asn Thr Phe Asp Gly Val Ala Asp Tyr Leu Gln

1 5 10 15

aca tat cat aag cta cct gat aat tac att aca aaa tca gaa gca caa 96

Thr Tyr His Lys Leu Pro Asp Asn Tyr Ile Thr Lys Ser Glu Ala Gln

20 25 30

gcc ctc ggc tgg gtg gca tca aaa ggg aac ctt gca gac gtc gct ccg 144

Ala Leu Gly Trp Val Ala Ser Lys Gly Asn Leu Ala Asp Val Ala Pro

35 40 45

ggg aaa agc atc ggc gga gac atc ttc tca aac agg gaa ggc aaa ctc 192

Gly Lys Ser Ile Gly Gly Asp Ile Phe Ser Asn Arg Glu Gly Lys Leu

50	55	60	
ccg ggc aaa agc gga cga aca tgg cgt gaa gcg gat att aac tat aca	240		
Pro Gly Lys Ser Gly Arg Thr Trp Arg Glu Ala Asp Ile Asn Tyr Thr			
65	70	75	80
tca ggc ttc aga aat tca gac cgg att ctt tac tca agc gac tgg ctg	288		
Ser Gly Phe Arg Asn Ser Asp Arg Ile Leu Tyr Ser Ser Asp Trp Leu			
85	90	95	
att tac aaa aca acg gac cat tat cag acc ttt aca aaa atc aga taa	336		
Ile Tyr Lys Thr Thr Asp His Tyr Gln Thr Phe Thr Lys Ile Arg			
100	105	110	
ggtaacc			343

<210> 2

<211> 112

<212> PRT

<213> Bacillus amyloliquefaciens

<400> 2

Met Val Pro Val Ile Asn Thr Phe Asp Gly Val Ala Asp Tyr Leu Gln	
1	5 10 15
Thr Tyr His Lys Leu Pro Asp Asn Tyr Ile Thr Lys Ser Glu Ala Gln	
20	25 30
Ala Leu Gly Trp Val Ala Ser Lys Gly Asn Leu Ala Asp Val Ala Pro	
35	40 45
Gly Lys Ser Ile Gly Gly Asp Ile Phe Ser Asn Arg Glu Gly Lys Leu	
50	55 60
Pro Gly Lys Ser Gly Arg Thr Trp Arg Glu Ala Asp Ile Asn Tyr Thr	
65	70 75 80
Ser Gly Phe Arg Asn Ser Asp Arg Ile Leu Tyr Ser Ser Asp Trp Leu	

85

90

95

Ile Tyr Lys Thr Thr Asp His Tyr Gln Thr Phe Thr Lys Ile Arg

100

105

110

&lt;210&gt; 3

&lt;211&gt; 342

&lt;212&gt; DNA

&lt;213&gt;

&lt;400&gt; 3

atggtaccgg ttattcaaca cgtttgacgg ggttgccgat tatcttcaga catatcataa 60  
 gctacctgat aattacatta caaaatcaga agcacaagcc ctccgctggg tggcatcaaa 120  
 agggaacctt gcagacgtcg ctccggggaa aagcatcggc ggagacatct tctcaaacag 180  
 ggaaggcaaa ctcccgggca aaagcggacg aacatggcgt gaagcggata ttaactatac 240  
 atcaggettc agaaattcag accggattct ttactcaagc gactggctga ttacaaaac 300  
 aacggaccat tatcagacct ttacaaaaat cagtaatcta ga 342

&lt;210&gt; 4

&lt;211&gt; 6548

&lt;212&gt; DNA

<213> Escherichia coli LE392

&lt;220&gt;

&lt;223&gt; Clone: pTS172

&lt;400&gt; 4

aattcaagct tgacgtcagg tggcactttt cggggaaatg tgcgcggaac ccctatttgt 60  
 ttatTTTTTct aaatacattc aaatatgtat ccgctcatga gacaataacc ctgataaatg 120  
 cttcaataat attgaaaaag gaagagtatg agtattcaac atttccgtgt cgcccttatt 180  
 ccctTTTTTtg cggcattttg ccttcctgtt tttgtcacc cagaaacgct ggtgaaagta 240

aaagatgctg aagatcagtt ggggtgcacga gtgggttaca tcgaactgga tctcaacagc 300  
 ggtaagatcc ttgagagttt tcgccccgaa gaacgttttc caatgatgag cactttttaa 360  
 gttctgctat gtggcgcggt attatcccgt attgacgccg ggcaagagca actcggtcgc 420  
 cgcatacact attctcagaa tgacttggtt gagtactcac cagtcacaga aaagcatctt 480  
 acggatggca tgacagtaag agaattatgc agtgctgcc aaccatgag tgataacact 540  
 gcggccaact tacttctgac aacgatcgga ggaccgaagg agctaaccgc ttttttgcac 600  
 aacatggggg atcatgtaac tcgccttgat cgttggggaac cggagctgaa tgaagccata 660  
 ccaaacgacg agcgtgacac cacgatgcct gtagcaatgg caacaacgtt gcgcaaacta 720  
 ttaactggcg aactacttac tctagcttcc cggcaacaat taatagactg gatggaggcg 780  
 gataaagttg caggaccact tctgcgctcg gcccttcggt ctggctggtt tattgctgat 840  
 aaatctggag ccggtgagcg tgggtctcgc ggtatcattg cagcactggg gccagatggt 900  
 aagccctccc gtatcgtagt tatctacacg acgggggagtc aggcaactat ggatgaacga 960  
 aatagacaga tcgctgagat aggtgcctca ctgattaagc attggtaact gtcagaccaa 1020  
 gtttactcat atatacttta gattgattta aaacttcatt ttttaattta aaggatctag 1080  
 gtgaagatcc tttttggctc gagtctcatg accaaaatcc cttaacgtga gttttcgttc 1140  
 cactgagcgt cagaccccggt agaaaagatc aaaggatctt cttgagatcc tttttttctg 1200  
 cgcgtaatct gctgcttgca aacaaaaaaa ccaccgctac cagcgggtgtt ttgtttgccg 1260  
 gatcaagagc taccaactct ttttccgaag gtaactggct tcagcagagc gcagatacca 1320  
 aatactgtcc ttctagtgtg gccgtagtta ggccaccact tcaagaactc tgtagcaccg 1380  
 cctacatacc tcgctctgct aatcctgtta ccagtggctg ctgccagtgg cgataagtcg 1440  
 tgtettaccg ggttggaactc aagacgatag ttaccggata aggcgcagcg gtcgggctga 1500  
 acgggggggtt cgtgcacaca gccagcttg gagcgaacga cctacaccga actgagatac 1560  
 ctacagcgtg agcattgaga aagcgccacg cttcccgaag ggagaaaggc ggacaggtat 1620  
 ccggtaaagc gcagggtcgg aacaggagag cgcacgaggg agcttccagg gggaaacgcc 1680  
 tggtatcttt atagtccgtt cgggtttcgc cacctctgac ttgagcgtcg atttttgtga 1740  
 tgctcgtcag gggggcggag cctatggaaa aacgccagca acgcggcctt tttacggttc 1800  
 ctggcctttt gctggccttt tgetcacatg ttctttcctg cgttatcccc tgattctgtg 1860  
 gataaccgta ttaccgcctt tgagttagct gataccgctc gccgcagccg aacgaccgag 1920  
 cgcagcgagt cagtgagcga ggaagcgga gagcgcccaa tacgcaaacc gcctctcccc 1980

gcgcgttggc	ctgatcagaa	ttcatatgca	cgtgttcccc	atctagtaac	atagatgaca	2040
ccgcgcgcga	taattttatcc	tagtttgccg	gctatatattt	gttttctatc	gcgtattaaa	2100
tgtataattg	cgggactcta	atcataaaaa	cccatctcat	aaataacgtc	atgcattaca	2160
tgtaattat	tacatgctta	acgtaattca	acagaaatta	tatgataatc	atcgcaagac	2220
cggcaacagg	attcaatctt	aagaaaacttt	attgccaaat	gtttgaacga	tctgcttcgg	2280
aggttacctt	atctgatttt	tgtaaaggtc	tgataatggg	ccgttgtttt	gtaaatcagc	2340
cagtcgcttg	agtaaagaat	ccggtctgaa	tttctgaagc	ctgatgtata	gttaatatcc	2400
gcttcacgcc	atgttcgtcc	gcttttgccc	gggagtttgc	cttccctgtt	tgagaagatg	2460
tctccgccga	tgtttttccc	cggagcgcag	tctgcaaggt	tcccttttga	tgccaccag	2520
ccgagggctt	gtgctttctga	ttttgtaatg	taattatcag	gtagcttatg	atatgtctga	2580
agataatccg	caaccccgtc	aaacgtgttg	ataaccggta	ccatcgcgac	ggcttgatgg	2640
atctcttgct	ggacaccggg	atgctaggat	gggttatcgt	ggccggcgtg	cgtgtgtggc	2700
ttttgtaggc	gccggcgcag	gcgggggcaa	tgtggcaggt	gagtcacggg	gcaagcgtgc	2760
gcaagtgact	gcaacaacca	aggacggtca	tggcgaaagc	acctcacgcg	tccaccgtct	2820
acaggatgta	gcagtagcac	ggtgaaagaa	gtgttgtccc	gtccattagg	tgcattctca	2880
ccgttggcc	gaacaggacc	gttcaacagt	taggttgagt	gtaggacttt	tacgtggtta	2940
atgtatggca	aatagtagta	aattttgccc	ccattgggtct	ggctgagata	gaacatatcc	3000
tggaaagcct	ctagcatatc	ttttttgaca	gctaaacttt	gcttcttgcc	ttcttggtct	3060
agcaatgacg	ttgcccatgt	cgtggcaaac	atctggtaag	gtaactgtat	tcgtttgttc	3120
ccttcaacgg	ctcaatcccc	acaggccaag	ctatcctttc	cttggcagta	taggctcctt	3180
gagagattat	actaccattt	ttaagtgcct	ataaagacga	tgctctctaa	ccagatcgat	3240
cagaaacaca	aagtttttagc	agcgtaatat	cccacacaca	tacacacacg	aagctatgcc	3300
tcctcatttt	ccgagagatt	ctgacagtga	ccagaatgtc	agaatgccat	ttcatgggca	3360
caagtcgac	cacaagcttc	ttggtggagg	tcaagggtgtg	ctattattat	tcgcttttcta	3420
ggaaattatt	cagaattagt	gccttttatc	ataactttctc	tctgagccga	tgtggtttttg	3480
gatttcattg	ttgggagcta	tgcagttgcg	gatattctgc	tgtggaagaa	caggaactta	3540
tctgcggggg	tccttgctgg	ggcaacattg	atatggttcc	tgttcgatgt	agtagaatac	3600
aatataattc	cgctcctttg	ccagattgcc	attcttgcca	tgcttgatgat	cttcatttgg	3660
tcaaatgccg	caccactctt	ggacaggtat	tagcttttatt	tcctgtggag	atggtagaaa	3720

actcagctta	cagaaatggc	atttcacgta	gtataacgca	agacattagg	tactaaaact	3780
caactaactg	tttccgaatt	tcaggggccc	tccaaggatc	ccagaaatca	tcattctctga	3840
acatgccttc	agagaaatgg	cattgaccgt	ccattacaaa	ctaacgtaca	ctgtatctgt	3900
tctttacgac	attgcatgtg	gaaaggatct	gaagagattt	ctcctgggtac	ataataatct	3960
actcctttgc	tacgttaata	agagatgtaa	aaacatgcaa	cagttccagt	gccaacattg	4020
tccaaggatt	gtgcaattct	ttctggagcg	ctaaaattga	ccagattaga	cgcacagaa	4080
tattgaattg	cagagttagc	caataatcct	cataatgtta	atgtgtctatt	gttggttact	4140
actcaatata	gttctggact	aacaatcaga	ttgtttatga	tattaagggtg	gttggatctc	4200
tattggtatt	gtcggcgatt	ggaagttctt	gcagcttgac	aagtctacta	tatatgtgta	4260
ggtattccag	ataaatatta	aattttaata	aaacaatcac	acagaaggat	ctgcggccgc	4320
tagcctaggc	cggggcccac	aaaaatctga	gcttaacagc	acagttgctc	ctctcagagc	4380
agaatcgggt	attcaacacc	ctcatatcaa	ctactacgtt	gtgtataacg	gtccacatgc	4440
cggtatatac	gatgactggg	gttgtacaaa	ggcggcaaca	aacggcggtc	cgggagttgc	4500
acacaagaaa	tttgccacta	ttacagaggc	aagagcagca	gctgacgcgt	acacaacaag	4560
tcagcaaaca	gacaggttga	acttcatccc	caaaggagaa	gctcaactca	agcccaagag	4620
ctttgctaag	gccctaacaa	gcccaccaaa	gcaaaaagcc	cactggctca	cgctaggaac	4680
caaaaggccc	agcagtgatc	cagccccaaa	agagatctcc	tttggcccgg	agattacaat	4740
ggacgatttc	ctctatcttt	acgatctagg	aaggaagttc	gaagggtgaag	gtgacgacac	4800
tatgttcacc	actgataatg	agaaggttag	cctcttcaat	ttcagaaaaga	atgctgaccc	4860
acagatggtt	agagaggcct	acgcagcagg	tctcatcaag	acgatctacc	cgagtaacaa	4920
tctccaggag	atcaaatacc	ttccaagaa	ggttaaagat	gcagtcaaaa	gattcaggac	4980
taattgcac	aagaacacag	agaaagacat	atttctcaag	atcagaagta	ctattccagt	5040
atggacgatt	caaggcttgc	ttcataaacc	aaggcaagta	atagagattg	gagtctctaa	5100
aaaggtagtt	cctactgaat	ctaaggccat	gcatggagtc	taagattcaa	atcgaggatc	5160
taacagaact	cgccgtgaag	actggcgaac	agttcataca	gagtctttta	cgactcaatg	5220
acaagaagaa	aatcttcgtc	aacatggttg	agcacgacac	tctggtctac	tcaaaaaatg	5280
tcaaagatac	agtctcagaa	gaccaaaggg	ctattgagac	ttttcaacaa	aggataattt	5340
cgggaaacct	cctcggattc	cattgcccag	ctatctgtca	cttcatcgaa	aggacagtag	5400
aaaaggaagg	tggtctctac	aaatgccatc	attgcgataa	aggaaaggct	atcattcaag	5460

atgcctctgc cgacagtggg cccaaagatg gacccccacc cacgaggagc atcgttgaaa 5520  
 aagaagacgt tccaaccacg tcttcaaagc aagtggattg atgtgacatc tccactgacg 5580  
 taagggatga cgcacaatcc cactatcctt cgcaagaccc ttcctctata taaggaagtt 5640  
 catttcattt ggagaggaca cgctgaaatc accagtctct ctctataaat ctatctctct 5700  
 ctctataacc atggacccag aacgacgccc ggccgacatc cgccgtgcca ccgaggcgga 5760  
 catgccggcg gtctgcacca tcgtcaacca ctacatcgag acaagcacgg tcaacttccg 5820  
 taccgagccg caggaaccgc aggagtggac ggacgacctc gtccgtctgc gggagcgcta 5880  
 tccctggctc gtcgccgagg tggacggcga ggctgccggc atgcctacg cgggccccctg 5940  
 gaaggcacgc aacgcctacg actggacggc cgagtgcacc gtgtacgtct cccccgccca 6000  
 ccagcggacg ggactgggct ccacgtctta caccacctg ctgaagtccc tggaggcaca 6060  
 gggcttcaag agcgtggtcg ctgtcatcgg gctgcccac gacccgagcg tgcgcatgca 6120  
 cgaggcgctc ggatatgccc cccgcggcat gctgcggcg gccggcttca agcacgggaa 6180  
 ctggcatgac gtgggtttct ggcagctgga cttcagcctg ccggtaccgc cccgtccggt 6240  
 cctgccccgc accgagatct gagatcacgc gttctaggat ccccgatga gctaagctag 6300  
 ctatatcatc aatttatgta ttacacataa tatcgactc agtctttcat ctacggcaat 6360  
 gtaccagctg atataatcag ttattgaaat atttctgaat ttaaacttgc atcaataaat 6420  
 ttatgttttt gcttggacta taatactga cttgttattt tatcaataaa tatttaaaact 6480  
 atatttcttt caagatggga attaacatct acaaattgcc ttttcttctc gaccatgtac 6540  
 gtatcgcg 6548

<210> 5

<211> 6539

<212> DNA

<213> Escherichia coli LE392

<220>

<223> Clone: pTS431

<400> 5

aattcaagct tgacgtcagg tggcactttt cggggaaatg tgcgcggaac ccctatttgt 60

ttatTTTTct	aaatacatTC	aaatatgtat	ccgctcatga	gacaataacc	ctgataaaatg	120
cttcaataat	attgaaaaag	gaagagtatg	agtattcaac	atttccgtgt	cgcccttatt	180
ccctTTTTtg	cggcatTTTTg	ccttcctgtt	tttgctcacc	cagaaacgct	ggtgaaagta	240
aaagatgctg	aagatcagtt	gggtgcacga	gtgggttaca	tcgaactgga	tctcaacagc	300
ggtaagatcc	ttgagagttt	tcgccccgaa	gaacgttttc	caatgatgag	cactttttaa	360
gttctgctat	gtggcgcggt	attatcccgt	attgacgccg	ggcaagagca	actcggtcgc	420
cgcatacact	attctcagaa	tgacttggtt	gagtactcac	cagtcacaga	aaagcatctt	480
acggatggca	tgacagtaag	agaattatgc	agtgctgcc	taaccatgag	tgataacact	540
gcggccaact	tactttctgac	aacgatcgga	ggaccgaagg	agctaaccgc	ttttttgcac	600
aacatggggg	atcatgtaac	tcgccttgat	cgttggggaac	cggagctgaa	tgaagccata	660
ccaaacgacg	agcgtgacac	cacgatgcct	gtagcaatgg	caacaacggt	gcgcaaacta	720
ttactggcgc	aactacttac	tctagcttcc	cggcaacaat	taatagactg	gatggaggcg	780
gataaaagttg	caggaccact	tctgcgctcg	gcccttccgg	ctggctgggt	tattgctgat	840
aaatctggag	ccggtgagcg	tgggtctcgc	ggtatcattg	cagcactggg	gccagatggt	900
aagccctccc	gtatcgtagt	tatctacacg	acggggagtc	aggcaactat	ggatgaacga	960
aatagacaga	tcgctgagat	aggtgcctca	ctgattaagc	attggtaact	gtcagaccaa	1020
gtttactcat	atatacttta	gattgattta	aaacttcatt	tttaatttaa	aaggatctag	1080
gtgaagatcc	tttttggtc	gagtctcatg	accaaaatcc	cttaacgtga	gttttcgttc	1140
cactgagcgt	cagaccccg	agaaaagatc	aaaggatctt	cttgagatcc	tttttttctg	1200
cgcgtaatct	gctgcttgca	aacaaaaaaa	ccaccgctac	cagcggtggt	ttgtttgccg	1260
gatcaagagc	taccaactct	ttttccgaag	gtaactggct	tcagcagagc	gcagatacca	1320
aatactgtcc	ttctagtgt	gccgtagtta	ggccaccact	tcaagaactc	tgtagcaccg	1380
cctacatacc	tcgctctgct	aatcctgtta	ccagtggctg	ctgccagtgg	cgataagtcg	1440
tgtcttaccg	ggttggactc	aagacgatag	ttaccggata	aggcgcagcg	gtcgggctga	1500
acgggggggtt	cgtgcacaca	gccagcttg	gagcgaacga	cctacaccga	actgagatac	1560
ctacagcgtg	agcattgaga	aagcgccacg	cttcccgaag	ggagaaaggc	ggacaggtat	1620
cgggtaagcg	gcagggtcgg	aacaggagag	cgcacgaggg	agcttccagg	gggaaacgcc	1680
tggtatcttt	atagtcctgt	cgggtttcgc	cacctctgac	ttgagcgtcg	atttttgtga	1740
tgctcgtcag	gggggcggag	cctatggaaa	aacgccagca	acgcggcctt	tttaocgttc	1800

ctggcctttt	gctggccttt	tgctcacatg	tcttttctg	cgttatcccc	tgattctgtg	1860
gataaccgta	ttaccgcctt	tgagtgagct	gataccgctc	gccgcagccg	aacgaccgag	1920
cgcagcgagt	cagtgagcga	ggaagcggaa	gagcgcccaa	tacgcaaacc	gcctctcccc	1980
gcgcgttggc	ctgatcagaa	ttcttcccga	tctagtaaca	tagatgacac	cgcgcgcgat	2040
aatttatcct	agtttgcgcg	ctatattttg	ttttctatcg	cgtattaaat	gtataattgc	2100
gggactctaa	tcataaaaac	ccatctcata	aataacgtca	tgcattacat	gttaattatt	2160
acatgcttaa	cgtaattcaa	cagaaattat	atgataatca	tcgcaagacc	ggcaacagga	2220
ttcaatctta	agaaacttta	ttgccaaatg	tttgaacgat	ctgcttcgga	tcctctagat	2280
tactgatttt	tgtaaaggtc	tgataatggg	ccgttgtttt	gtaaatcagc	cagtcgcttg	2340
agtaaagaat	ccggctctgaa	tttctgaagc	ctgatgtata	gttaatatcc	gcttcacgcc	2400
atgttcgtcc	gcttttgccc	gggagtttgc	cttccctgtt	tgagaagatg	tctccgccga	2460
tgcttttccc	cggagcgcac	tctgcaaggt	tcccttttga	tgccaccag	ccgagggctt	2520
gtgcttctga	ttttgtaatg	taattatcag	gtagcttatg	atatgtctga	agataatccg	2580
caaccccgtc	aaacgtgttg	aataaccggt	accatcgcca	cggcttgatg	gatctcttgc	2640
tggaacaccg	gatgctagga	tgggttatcg	tggccggcgt	gcgtgtgtgg	cttttgtagg	2700
cgccggcgac	ggcgggggca	atgtggcagg	tgagtcacgg	tgcaagcgtg	cgcaagtgc	2760
tgcaacaacc	aaggacggtc	atggcgaaaag	cacctcacgc	gtccaccgtc	tacaggatgt	2820
agcagtagca	cggtgaaaga	agtgttgctc	cgtccattag	gtgcattctc	accgttggcc	2880
agaacaggac	cgttcaacag	ttaggttgag	tgtaggactt	ttacgtgggt	aatgtatggc	2940
aaatagtagt	aaattttgcc	cccattggtc	tggttgagat	agaacatatt	ctggaaagcc	3000
tctagcatat	cttttttgac	agctaaactt	tgtttcttgc	cttcttggtc	tagcaatgac	3060
gttgcccatg	tcgtggcaaa	catctggtaa	ggtaactgta	ttcgtttgtt	cccttcaacg	3120
gctcaatccc	cacaggccaa	gctatccttt	ccttggcagt	ataggctcct	tgagagatta	3180
tactaccatt	tttaagtgt	tataaagacg	atgctctcta	accagatcga	tcagaaacac	3240
aaagttttag	cagcgtaata	tcccacacac	atacacacac	gaagctatgc	ctcctcattt	3300
tccgagagat	tctgacagtg	accagaatgt	cagaatgcca	tttcatgggc	acaagtcgat	3360
ccacaagctt	cttggtggag	gtcaaggtgt	gctattatta	ttcgctttct	aggaaattat	3420
tcagaattag	tgccttttat	cataacttct	ctctgagccg	atgtggtttt	ggatttcatt	3480
gttggggagct	atgcagttgc	ggatattctg	ctgtggaaga	acaggaactt	atctgcgggg	3540

gtccttgctg gggcaacatt gatatggttc ctgttcgatg tagtagaata caatataatt 3600  
 ccgctccttt gccagattgc cattcttgcc atgcttgtga tcttcatttg gtcaaatgcc 3660  
 gcaccactct tggacaggta ttagctttat ttctgttgga gatggtagaa aactcagctt 3720  
 acagaaatgg catttcacgt agtataacgc aagacattag gtactaaaac tcaactaact 3780  
 gtttccgaat ttcaggggcc ctccaaggat cccagaaatc atcatctctg aacatgcctt 3840  
 cagagaaatg gcattgaccg tccattacaa actaacgtac actgtatctg ttctttacga 3900  
 cattgcatgt ggaaaggatc tgaagagatt tctccttgga cataataatc tactcctttg 3960  
 ctacgttaat aagagatgta aaaacatgca acagttccag tgccaacatt gtccaaggat 4020  
 tgtgcaattc tttctggagc gctaaaattg accagattag acgcatcaga atattgaatt 4080  
 gcagagtiag ccaataatcc tcataatgtt aatgtgctat tgttgttcac tactcaatat 4140  
 agttctggac taacaatcag attgtttatg atattaaggt ggttggatct ctatttggtat 4200  
 tgtcggcgat tggaagttct tgcagcttga caagtctact atatattggg aggtattcca 4260  
 gataaatatt aaattttaat aaaacaatca cacagaagga tctgcggccg ctagcctagg 4320  
 cccgggceca caaaaatctg agcttaacag cacagttgct cctctcagag cagaatcggg 4380  
 tattcaacac cctcatatca actactacgt tgtgtataac ggtccacatg ccggtatata 4440  
 cgatgactgg ggttgtacaa aggcggcaac aaacggcggt cccggagtig cacacaagaa 4500  
 atttgccact attacagagg caagagcagc agctgacgcg tacacaacaa gtcagcaaac 4560  
 agacaggttg aacttcatcc ccaaaggaga agctcaactc aagccaaga gctttgctaa 4620  
 ggccctaaca agcccaccaa agcaaaaagc ccactggctc acgctaggaa ccaaaaggcc 4680  
 cagcagtgat ccagcccaa aagagatctc ctttgccccg gagattacaa tggacgattt 4740  
 cctctatctt tacgatctag gaaggaagtt cgaaggtgaa ggtgacgaca ctatgttcac 4800  
 cactgataat gagaaggtta gcctcttcaa tticagaaag aatgctgacc cacagatggt 4860  
 tagagaggcc tacgcagcag gtctcatcaa gacgatctac ccgagtaaca atctccagga 4920  
 gatcaaatac ctcccaaga aggttaaaga tgcagtcaaa agattcagga ctaattgcat 4980  
 caagaacaca gagaaagaca tattttctca gatcagaagt actattccag tatggacgat 5040  
 tcaaggcttg cttcataaac caaggcaagt aatagagatt ggagtctcta aaaaggtagt 5100  
 tcctactgaa tctaaggcca tgcattggagt ctaagattca aatcgaggat ctaacagaac 5160  
 tcgccgtgaa gactggcgaa cagttcatac agagtctttt acgactcaat gacaagaaga 5220  
 aaatcttcgt caacatgggtg gagcacgaca ctctgggtcta ctccaaaaat gtcaagata 5280

cagtctcaga agaccaaagg gctattgaga cttttcaaca aaggataatt tcgggaaacc 5340  
 tcctcggatt ccattgccca gctatctgtc acttcatcga aaggacagta gaaaaggaag 5400  
 gtggctccta caaatgccat cattgcgata aaggaaaggc tatcattcaa gatgcctctg 5460  
 ccgacagtgg tcccaaagat ggacccccac ccacaggag catcgtggaa aaagaagacg 5520  
 ttccaaccac gtcttcaaag caagtggatt gatgtgacat ctccactgac gtaagggatg 5580  
 acgcacaate ccactatcct tcgcaagacc cttcctctat ataaggaagt tcatttcatt 5640  
 tggagaggac acgtgaaat caccagtctc tctctataaa tctatctctc tctctataac 5700  
 catggacca gaacgacgcc cggccgacat ccgccgtgcc accgaggcgg acatgccggc 5760  
 ggtctgcacc atcgtcaacc actacatcga gacaagcacg gtcaacttcc gtaccgagcc 5820  
 gcaggaaccg caggagtggg cggacgacct cgtccgtctg cgggagcgct atccctggct 5880  
 cgtcgccgag gtggacggcg aggtcgccgg catcgccctac gcggggcccct ggaaggcacg 5940  
 caacgcctac gactggacgg ccgagtcgac cgtgtacgtc tcccccgcc accagcggac 6000  
 gggactgggc tccacgtctt acaccacct gctgaagtcc ctggaggcac agggcttcaa 6060  
 gagcgtggtc gctgtcatcg ggctgccccaa cgaccgagc gtgcgcatgc acgaggcgct 6120  
 cggatatgcc ccccgcgga tgctgcgggc ggccggcttc aagcacggga actggcatga 6180  
 cgtgggtttc tggcagctgg acttcagcct gccgggtaccg ccccgctcgg tcctgcccgt 6240  
 caccgagatc tgagatcacg cgttctagga tccccgatg agctaagcta gctatatcat 6300  
 caatttatgt attacacata atatcgact cagtctttca tctacggcaa tgtaccagct 6360  
 gatataatca gttattgaaa ttttctgaa tttaaacttg catcaataaa tttatgtttt 6420  
 tgcttggact ataatacctg acttgttatt ttatcaataa atatttaaac tatatttctt 6480  
 tcaagatggg aattaacatc tacaaattgc cttttcttat cgaccatgta cgtatcgcg 6539

<210> 6

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer 1

<400> 6

cgttcggctc gatggtaccg gttatcaaca cgtttga

37

<210> 7

<211> 38

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer 2

<400> 7

cctctagatt atctgatttt tgtaaaggtc tgataatg

38